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Interspecies interactions between *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* SAG 86.81 in a co-cultivation system at various growth phases

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ABSTRACT

In lakes, cyanobacterial blooms are frequently associated with green algae and dominate the phytoplankton community in successive waves. In the present study, the interactions between *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* were studied to clarify the probable ecological significance of algal secondary metabolites; focusing on the role of cyanotoxin 'microcystin-LR' (MC-LR). A dialysis co-cultivation technique was applied where *M. aeruginosa* was grown inside and *D. subspicatus* was cultured outside of the dialysis tubing. The concentration of the intra- and extracellular MC-LR and the growth of two species were measured at different time points over a period of one month. Additionally, the growth of the two species in the culture filtrate of one another and the effect of the purified MC-LR on the growth of the green alga were studied. The results indicated that the co-existing species could affect each other depending on the growth phases. Despite the early dominance of *D. subspicatus* during the logarithmic phase, *M. aeruginosa* suppressed the growth of the green alga at the stationary phase, which coincided with increased MC production and release. However, the inhibitory effects of *Microcystis* might be related to its other extracellular metabolites rather than, or possibly in addition to, MC.

1. Introduction

Monitoring the composition of the phytoplankton populations has shown that algal species undergo a sequence of dominance; a phenomenon called seasonal succession (Reynolds, 1980). According to the seasonal pattern, diatoms (Diatomophyceae) dominate the phytoplankton community during the winter and spring, whereas during the summer green algae (Chlorophyceae) prevail, and in the late summer and autumn cyanobacteria outcompete their predecessors (Sommer, 1989). However, under environmental parameters favouring algal growth such as high nutrient availability (primarily nitrogen and phosphorous) in eutrophic waters, abundant sunlight, warm water temperature (>25 °C), and stagnant water, some species of cyanobacteria grow explosively and form large blooms outside of their typical season (Paerl and Otten, 2013; Rastogi et al., 2015; Scholz et al., 2017). Additionally, eutrophication of lakes and climate change influence the algal seasonal pattern, favouring the formation of harmful cyanobacterial blooms. The occurrence of toxic cyanobacterial blooms, which have undesirable effects on humans, animals, and aquatic biota, has been reported in many countries throughout the world (Zanchett and Oliveira-Filho, 2013; Svirčev et al., 2017).

Understanding the factors that induce the shift in the phytoplankton composition to the domination of a toxic bloom holds many advantages, especially concerning water quality, treatment, and governance. Recent studies reported that the seasonal fluctuation of phytoplankton species is influenced not only by the environmental factors (Chen et al., 2003; Karadžić et al., 2013; Yang et al., 2018) but also by the interspecies interactions (Sukenik et al., 2002; Vardi et al., 2002; Legrand et al., 2003; Leão et al., 2009; Chia et al., 2018). In freshwater ecosystems, cyanobacterial blooms influence the composition of microbial communities and the co-occurrence patterns of eukaryotic plankton (Xue et al., 2018; L. Liu et al., 2019; M. Liu et al., 2019).

Abbreviations: MCs, Microcystins; MeOH, Methanol; TFA, Trifluoroacetic acid; ACN, Acetonitrile

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Microalgae, including cyanobacteria and eukaryotic algae, are known to produce and release a great variety of secondary metabolites during their life cycles or by cell lysis (Leflaive and Ten-Hage, 2007) as a result of natural senescence or chemical treatment. Cyanotoxins, produced by cyanobacteria, are a broad range of toxic secondary metabolites (Sivonen, 2009). Among them, microcystins (MCs), which are synthesised non-ribosomally via a large multifunctional enzyme MC synthetase, are the most widely studied cyanotoxins (Nishizawa et al., 2000; Sivonen, 2009). The past studies have mainly focused on the toxicity of MCs while their probable natural functions are not well understood. It is also not yet clear why cyanobacteria pay such a high energetic price for MC synthesis and what advantages the toxin lends to the producer.

A large volume of significant work has already invested in understanding the physiological and ecological roles of MCs (as reviewed by Omidi et al. (2017)). It has been found that MCs could interfere in the photosynthesis and nutrient metabolism, iron uptake, quorum sensing, benthic survival and recruitment process, bloom maintenance, protection against the oxidative stress, defence against the grazers, and the interspecies interactions. In this regard, the ecological significance of MCs in aquatic ecosystems has been more elucidated by the studies of the toxin-related interactions between *Microcystis* spp. and other aquatic co-existing species (Vardi et al., 2002; Li and Li, 2012; Yang et al., 2014; Bittencourt-Oliveira et al., 2015).

Cyanobacterial blooms are frequently associated with green algae and together dominate the aquatic environments in successive waves (Sedmak and Kosi, 1998; Chen et al., 2003; Harel et al., 2013). Therefore, a study of the interactions between these two environmentally co-existing organisms, cyanobacteria and green algae, might provide more insights into the seasonal variation dynamics of phytoplankton populations.

In the present study, the interactions between the most common species to cause toxic cyanobacterial blooms, *Microcystis aeruginosa* PCC 7806, and a common freshwater green alga, *Desmodesmus subspicatus* SAG 86.81 were studied. To achieve this, we designed a co-cultivation system where two populations were growing together in close proximity but separated physically using dialysis tubing to study the effects of the co-growing species on each other in a continuous exposure mode through diffusible secondary metabolites beyond their physical cell-cell contacts. As a result, the changes in the growth of both species and MC-LR contents of *M. aeruginosa* were assessed. Moreover, the growth of the two species in the cell-free spent medium of each other was monitored. The green alga was exposed to different concentrations of the extracted MC-LR as well. Based on the results, the possible ecological significance of cyanobacterial secondary metabolites, with particular reference to MC-LR, in interspecies interactions, as well as the possibility of the production of secondary metabolites by green algae to influence the toxic cyanobacteria species, was considered.

2. Theory

Microcystins (MCs) are the most commonly studied cyanotoxins. Recently, the significance of MCs to the producing organisms beyond their toxicity gained particular attention. In an attempt to improve this knowledge, we applied a co-cultivation system in the present study, i.e. using a dialysis membrane to study the effects of the probable diffusible secondary metabolites of these two organisms on one another, concurrently excluding the effects of mixed-cultivation. To our knowledge, this is the first study to consider the efficiency of dialysis tubing in the interspecies interaction studies through the measurements of MC-LR on both sides of the tubing. This method may provide a more practical approach and information for future laboratory and field studies of microbial interactions.

3. Materials and methods

3.1. Algal monocultures and culture conditions

The cyanobacterium, *Microcystis aeruginosa* PCC 7806, was provided by the Pasteur Culture Collection of Cyanobacteria (PCC) (Paris, France) and the green alga, *Desmodesmus subspicatus* SAG 86.81 (formerly *Scenedesmus subspicatus*), was obtained from the SAG Culture Collection of Algae (Sammlung von Algenkulturen) (University of Göttingen, Germany). The species were cultivated sterile BG 11 medium (Rippka et al., 1979), which was adjusted to pH of 8 using 1 N HCl and 1 N NaOH, respectively. The cultures were incubated at 24 ± 1 °C under 12:12 light: dark cycle, with a light intensity of 2220 lm m^{-2} provided by cool white fluorescent irradiation. The cultures were grown for ten days to mid-exponential growth phase to use as inoculum in the co-cultivation experiments.

3.2. Setting up the co-cultivation system

The dialysis membranes (molecular weight cut-off (MWCO) 12–14 kDa; diameter of 29 mm; Spectra/Por®, Spectrum Laboratories, USA) were cut into 30 cm lengths before being soaked in distilled water for 15 min, incubated in 10 mM sodium bicarbonate ($NaHCO_3$) for 30 min at 80 °C, and then soaked in 10 mM Na_2EDTA for 30 min. The membranes were then placed in distilled water and autoclaved for 10 min at 121 °C. The prepared sterilised dialysis bags with an open top and a closed bottom end were each positioned aseptically in a 250 mL Schott bottle containing 180 mL fresh BG 11 medium.

M. aeruginosa PCC 7806 was transferred into the dialysis tubing containing 20 mL BG 11 medium and therefore the cell density at the start of the experiment was 1×10^7 cells/mL. *D. subspicatus* was inoculated into the bottle containing the dialysis tubing but outside of the tubing, which contained 180 mL of BG 11. The starting cell density of the green alga then amounting to 1×10^6 cells/mL (Fig. 1). Two controls were set up, i.e. one where *M. aeruginosa* was cultured in a dialysis tube without *D. subspicatus* on the outside (control 1, monoculture of *M. aeruginosa*) and the second where the green alga was cultured in a glass bottle medium containing a dialysis tube filled only with BG 11 medium without *M. aeruginosa* (control 2, monoculture of *D. subspicatus*) (Fig. 1). The co-cultivation experiments were evaluated at three phases in the life cycle of the microalgae, i.e. after the first, second, and fourth weeks representing the early logarithmic phase, the logarithmic phase, and the late logarithmic and stationary phases of growth, respectively. In other words, the treatments were prepared in triplicate for three

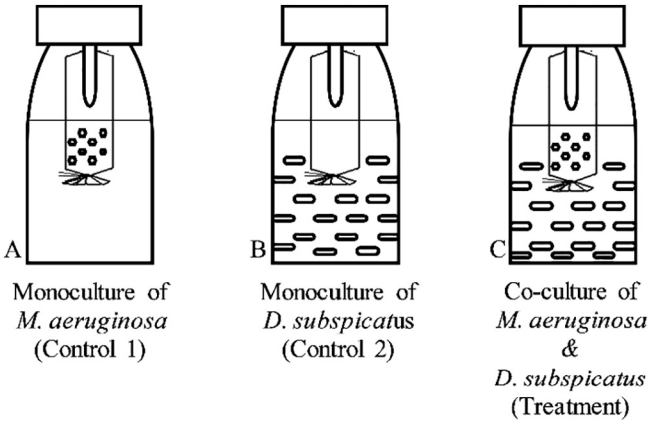

Fig. 1. A schematic view of the experimental design: A) monoculture of *M. aeruginosa* PCC 7806 in the dialysis tubing (control 1), B) monoculture of *D. subspicatus* SAG 86.81 out of the dialysis tubing (control 2), and C) a co-cultivation system containing *M. aeruginosa* PCC 7806 inside and *D. subspicatus* SAG 86.81 outside of the dialysis bag (treatment).

incubation periods, i.e. one, two, and four weeks, after which time samples were collected for cell counting and MC-LR analysis. The prepared cultures were incubated under the same conditions as described for the initial cultivation on a rotary shaker at 75 rpm at 23 ± 2 °C. Every five days, 5 mL of fresh, sterilised medium was added on the outside of the membranes under aseptic conditions to eliminate the possibility of competition for nutrients. At the end of the first, second and fourth weeks, respectively, 1 mL was sampled from the inside and outside of dialysis tubing from each replicate. Taking into account, a dilution factor of 10 was applied to the reported biomass of *Microcystis,* regarding the ratio of the volume of the culture medium inside of the dialysis tubing to the whole medium (20, 200).

3.3. Growth of algal species in cell-free spent medium

Cell-free spent medium of each species was obtained from the algal monocultures at logarithmic phase of growth after centrifugation (4000 ×*g*, 30 min, 4 °C). Then, 100 mL of culture media containing different percentages of spent medium (25, 50, 75, and 100) was prepared using fresh BG 11 medium. The target species each at the initial density of 5×10^5 cells/mL was inoculated, and the prepared cultures (five replicates) were incubated under the described culture conditions of the algal monocultures for one, two and three weeks. Finally, 1 mL of sample was collected at the end of the incubation periods for cell counting.

3.4. Exposure of the green alga to the extracted MC-LR

MC-LR was extracted from 25 mg of the lyophilised *Microcystis* biomass as described below in Section 3.6. The toxin concentration was determined using LC-MS/MS, as described Section 3.8. Then, 100 mL of culture media containing different concentrations of the extracted MC-LR (70, 140, 270, 410 and 520 µg/L) was prepared in 300 mL Erlenmeyer flasks. *D. subspicatus* at an initial density of 1×10^5 cells/mL (five replicates) were grown under the previously described monoculture conditions. The experiment was run for two weeks, and every 2 or 3 days 1 mL sample was taken for the growth measurements.

3.5. Growth measurement

The cell densities of *M. aeruginosa* and *D. subspicatus* were monitored by counting the number of cells using a haemocytometer (Neubauer) and light microscopy (Olympus CH-2).

3.6. Intracellular MC-LR extraction

Microcystis cells were harvested by centrifugation (4000 ×*g*, 30 min, 4 °C) of 5 mL sample, which was taken from the inside of the dialysis tubing. The resulting pellet was lyophilised using an LIO-5P (5 Pascal, Italy) freeze dryer. The cells of 3 mg freeze-dried biomass were disrupted in an ultrasonic bath (Allpax, Germany) for 15 min in 3 cycles of 5 min. Then, 1 mL of 70% methanol (MeOH) acidified with 0.1% trifluoroacetic acid (TFA) was added, and the mixture was continuously shaken (Intelli-mixer, neoLab®) for 1 h. The resulting slurry was centrifuged (10,000 ×*g*, 10 min, 4 °C), whereafter the supernatant was collected, and the pellet re-extracted following the same method. The procedure was repeated four times. Then, the resulting supernatants were combined and dried at 30 °C using a Concentrator Plus (Eppendorf, Germany). Finally, the residue was dissolved in 1 mL of 100% MeOH and centrifuged (20,800 ×*g*, 15 min, and 4 °C). The supernatant was stored at −20 °C until quantification with liquid chromatography-tandem mass spectroscopy (LC-MS/MS).

3.7. Extracellular MC-LR preparation

The concentration of extracellular MC-LR was measured both inside and outside of the dialysis tubing at the end of the first, second, and fourth incubation weeks in control 1 (monoculture of *M. aeruginosa*) and treatments. From 5 mL of sample collected from the inside of dialysis tubing, *Microcystis* cells were harvested by centrifugation (4000 ×*g*, 30 min, 4 °C), and the supernatant was filtered through a 0.22 µm Whatman filters (Sigma-Aldrich). From 30 mL sample collected from the outside of the dialysis membrane, the supernatant was collected after centrifugation (4000 ×*g*, 15 min, 4 °C) and then was filtered through a 0.22 µm Whatman filters (Sigma-Aldrich). The cell-free supernatants which have been collected from the inner and outer sides of the dialysis membrane were applied to a solid phase extraction column (Sep-Pak tC18 6 cc Vac Cartridge, 500 mg Sorbent per Cartridge, pore size 125 Å, particle size 37–55 µm, Waters). All columns were preconditioned with 10 mL of 100% MeOH, followed by 10 mL of distilled water both at a flow rate of approximately 5 mL/min. Sample loading and elution with 10 mL MeOH 100% were conducted at a flow rate of 1 mL/min. Extracts were evaporated in a Concentrator Plus (Eppendorf, Germany) at 30 °C. The resulting precipitates were solubilised in 1 mL of MeOH 100% and stored at −20 °C until quantification. The outer membrane MC content was divided by the inner membrane MC-LR concentration (O/I: Outer/Inner) to obtain the diffusion rate.

3.8. MC-LR quantification

Determination and quantification of MC-LR were carried out on an Alliance 2695 UHPLC coupled to a Micromass Quattro micro™, (Waters, Milford, MA, USA). The chromatographic separation of the samples was carried out on a reverse phase column using a Kinetex™ C18 column (2.1 × 50 mm, 2.6 µm pore size, Phenomenex). The column temperature was thermostated at 40 °C. The mobile phase consisted of solution A (MS-grade water containing 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN)) and solution B (ACN containing 0.1% TFA) at a flow rate of 0.2 mL/min. The linear gradient conditions of solutions were as follows: 0 min 100% A; 3.75–7 min 35% A, and 7.8–12 min 100% A. The injection volume was 20 µL. The elution peak of MC-LR was observed at 7.95 min.

For the tandem mass spectroscopy, the electrospray ionisation (ESI) conditions were set as follows: capillary voltage of 3 kV, source temperature of 120 °C, desolvation temperature of 500 °C, and cone gas flow-rate of 100 L/h. The collision energy was 65 V, and the cone voltage was 60 V. Desolvation gas flow-rate was 1000 L/h. Nitrogen was used as trigger gas and Argon as the collision gas. Parent compound and its fragment ions, respectively, were scanned at the following mass-to-charge ratio (m/z) 995.5 → 135.1 for MC-LR. Limit of MC-LR detection was set at 1 ng/mL and limit of quantification at 5 ng/mL (signal to noise S/N > 5). Standard solution of purified solid MC-LR (Enzo, Germany) in MeOH 100% was used to set the calibration curve.

3.9. Statistical analysis

Statistical analyses were performed for the cell numbers and MC contents using SPSS, version 24. Shapiro-Wilk test and Levene test were used to verify the normality of the data and homogeneity of variance, respectively. The observed data were assessed statistically by one-way analyses of variance (ANOVA) for differences between the mono- and co-cultures ($p \leq 0.05$). The Turkey HSD analysis and Student's *t*-test were used to assess the differences between means, if variables were homogeneous or heterogeneous, respectively. Data, which did not follow a normal distribution (the intracellular MC-LR in co-cultures at the end of the second week and the diffusion rate of extracellular MC-LR in co-cultures at the end of the fourth week) was analysed with non-parametric tests, such as the Kruskal-Wallis and Mann-Whitney-*U* test. All data were presented as mean ± standard deviation ($n = 3$). The Dunnett test and Student's t-test was used to assess the differences between means of algal biomass in cell-free spent media and the growth of green alga which was exposed to the extracted MC-LR, if variables were

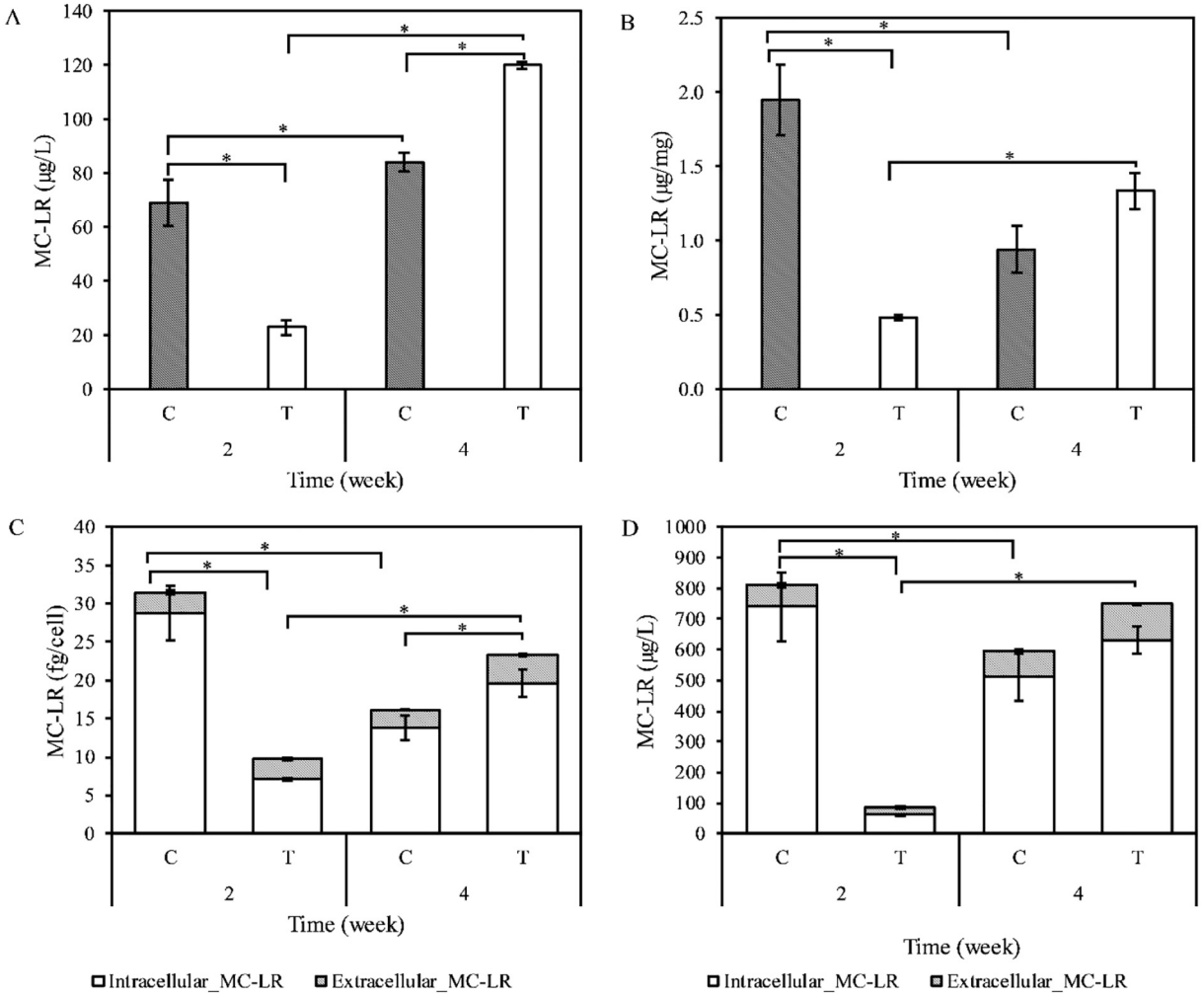

Fig. 2. The concentration of A) extracellular MC-LR (µg/L), B) intracellular MC-LR (µg/mg dry weight), C) MC-LR (intra- and extracellular) concentration per cell (fg/cell), and D) total MC-LR (intra- and extracellular) content (µg/L) in mono- and co-cultures at the end of the 2nd and the 4th week (C: control (monoculture); T: treatment (co-culture)). Data represent mean values ± standard deviation ($n = 3$). Significant differences observed at $p \leq 0.05$ (*).

homogeneous or heterogeneous, respectively. Data were displayed as means ± standard errors ($n = 5$). P value < 0.05 was set for statistical significance.

4. Results

4.1. MC concentration in co-cultivation experiments

After the first week of co-cultivation, i.e. during the early logarithmic phase of growth, neither intracellular nor extracellular MC-LR was detected in either monoculture (control 1) or simultaneous co-cultures (treatment). Over time, towards the end of the second week, i.e. during the logarithmic phase of growth, less total MC, which is the sum of intracellular and extracellular MC-LR, was detected in the co-cultivated (Fig. 2C and D, $p < 0.05$). In co-cultures, both the extracellular and intracellular MC-LR were significantly lower compared to monocultures ($p < 0.05$, Fig. 2A and B, respectively). However, the difference between intracellular MC-LR achieved with mono- and co-cultivated was greater than that seen with the extracellular MC-LR ($p < 0.05$, Fig. 2A and B). In co-cultures, the concentration of extracellular MC-LR (µg/L) was three-fold less than the monocultures while the content of the intracellular MC-LR (µg/mg dry weight) was four-fold less than the monoculture ($p < 0.05$, Fig. 2A and B, respectively). The results indicated that the MC release from cells was constant ($p > 0.05$) while the amount of intracellular MC-LR per cell was 4-fold decreased in co-cultures compared to monocultures (Fig. 2C, $p < 0.05$).

After the fourth week, which was during the late logarithmic and stationary phases of growth, in the treatments, total MC content (the sum of intracellular and extracellular MC-LR (µg/L)) had not changed compared to the simultaneously conducted monoculture ($p > 0.05$, Fig. 2D). Thus, the amount of extracellular MC-LR was elevated ($p < 0.05$, Fig. 2A) while the concentration of intracellular toxin was at the same level as the monocultures ($p > 0.05$, Fig. 2B). However, the total MC content per cell was higher compared to the simultaneous monoculture. The results indicated a significant elevation in the concentration of both intracellular and extracellular MC-LR per cell ($p < 0.05$, Fig. 2C).

The results showed that the dynamic variation of MC content was different in mono- and simultaneous co-cultures. In the presence of the green alga, with time, the increased release of MC into the surrounding media was higher ($p < 0.05$, Fig. 2A). From the second to the fourth week, in the monocultures, the extracellular MC-LR continuously increased from 69.3 µg/L after the second week to 84.3 µg/L after the fourth week. While, in the mixed cultures at the end of the second week, the extracellular MC-LR concentration was one-third of that measured with the monoculture (22.9 µg/L) which reached the highest quantified level (119.95 µg/L) ($p < 0.05$, Fig. 2A). Conversely, from the second to the fourth week, the concentration of intracellular MC-LR was significantly decreased with monoculture ($p < 0.05$) while it was

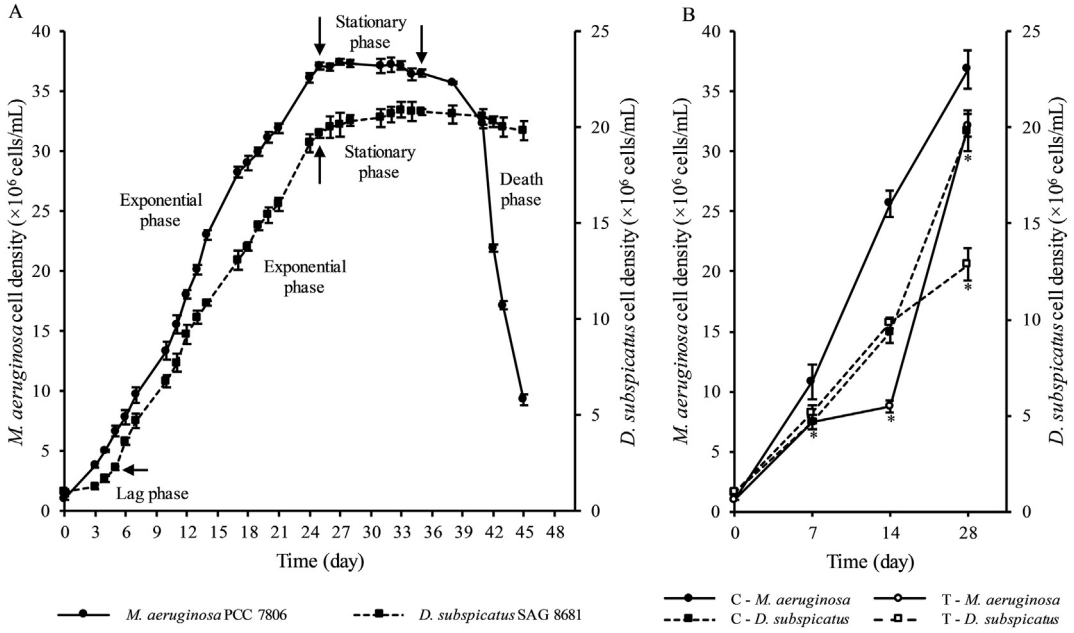

Fig. 3. Microalgal growth: A) the growth curves of *M. aeruginosa* PCC 7806 and *D. subspicatus*, and B) the growth of *M. aeruginosa* PCC 7806 and *D. subspicatus* in the co-cultivation system in mono- (controls) and co-cultures (treatments) (C: control, T: treatment). Data represent mean values ± standard deviation ($n = 3$). Significant differences were accepted at a *p*-value of $p < 0.05$ (*).

increased in co-cultures ($p < 0.05$, Fig. 2B). The highest level of intracellular MC-LR (2.0 μg/mg dry weight) was quantified in the monocultures after the second week; then over time from the second to the fourth week, it was reduced by half ($p < 0.05$, Fig. 2B). In contrast, the lowest level of intracellular MC-LR (0.5 μg/mg dry weight) was quantified in co-cultures after the second week, which had been raised approximately three-fold over time to the fourth week ($p < 0.05$, Fig. 2B).

4.2. Growth measurements

4.2.1. Growth of algal species in the co-cultivation experiment

The growth curves of *M. aeruginosa* and *D. subspicatus* in single were studied and documented prior to the co-cultivation experiments (Fig. 3A). The results showed that the growth of the two species was different when growing together (Fig. 3B). At the end of the first week of co-cultivation, no changes in the growth of the green alga were observed ($p > 0.05$) while the growth of *Microcystis* was remarkably inhibited ($p < 0.05$). Over time to the end of the second week, the growth of *Microcystis* was reduced to one third which was at the same level as the treatment of the first week ($p > 0.05$) and significantly lower than its simultaneous monocultures ($p < 0.05$) whereas the growth of *D. subspicatus* was not altered ($p > 0.05$). Finally, despite the significant inhibition of the growth of *Microcystis* during the second week, the *Microcystis* biomass was increased from the second to the end of the fourth week of co-cultivation. However, at the end of the fourth week, it was still significantly lower than the monocultures ($p < 0.05$, Fig. 3B). In contrast, the growth of *D. subspicatus* has been notably inhibited at the end of the fourth week ($p < 0.05$, Fig. 3B).

4.2.2. Growth of algal species in cell-free spent medium

After the first week, no changes in the growth of *Microcystis* was observed at different percentages of the spent medium of the green alga. Afterwards, towards the end of the experiment, the biomass in 25% enriched medium was at the same level as the control ($p > 0.05$) while at the higher percentages of the filtrate, the biomass was significantly reduced ($p < 0.05$, Fig. 4A).

After the first week, the growth of the green alga at different percentages of the *Microcystis* filtrate stayed unchanged compared to the control ($p > 0.05$, Fig. 4B). After the second week, the growth was inhibited at 75% and 100% filtrate ($p < 0.05$) while at the lower percentages of the filtrate, the biomass was not altered ($p > 0.05$). Towards the end of the experiment, the biomass of the green alga was significantly reduced ($p < 0.05$) at the higher levels of the spent medium (50, 75, and 100%) while it was not changed at 25% filtrate, compared to the control ($p > 0.05$, Fig. 4B).

4.2.3. Growth of the green alga which was exposed to the extracted MC-LR

After ten days, the density of the green alga at the lower concentrations of MC-LR (70, 140, and 270 μg/L) was at the same level as the control ($p > 0.05$) while increased concentrations of MC-LR (410 and 520 μg/L) significantly inhibited the growth of green alga ($p < 0.05$) (Fig. 5). Afterwards, on days 12 and 14, the density of *D. subspicatus* remained unchanged at the concentrations 70 and 140 μg/L ($p > 0.05$) while it was significantly reduced at the greater concentrations of MC-LR, compared to the control ($p < 0.05$, Fig. 5).

4.3. The efficiency of the dialysis membrane in co-cultivation experiments

The efficiency of the dialysis membrane used in the co-cultivation system was considered by measuring the concentration of the extracellular MC-LR at both sides of the dialysis tubing for both the controls and treatments (Fig. 6). In all cultures, the concentration of extracellular MC-LR on the inside of dialysis tubing was significantly higher than the outside ($p < 0.05$, Fig. 6). The only exception was seen for the co-cultures during the second week where the extracellular MC-LR diffused through the membrane and was equally dispersed between the two sides, amounting to 52.29 ± 4.54% MC-LR on the inside and 47.71 ± 4.54% on the outside of the dialysis tubing ($p > 0.05$, Figs. 6 and 7A). For the monoculture sampled after the fourth week, the co-culture after the fourth week, and the monoculture after the second week, the diffusion rates were not equal, i.e. with the diffusion rates of 0.6, 0.5 and 0.2, respectively (Fig. 7B).

In the co-cultures, after the second week, where the minimum concentration of total extracellular MC-LR (22.9 μg/L) was quantified, the highest MC diffusion rate (0.9) was observed (Fig. 7B). After the

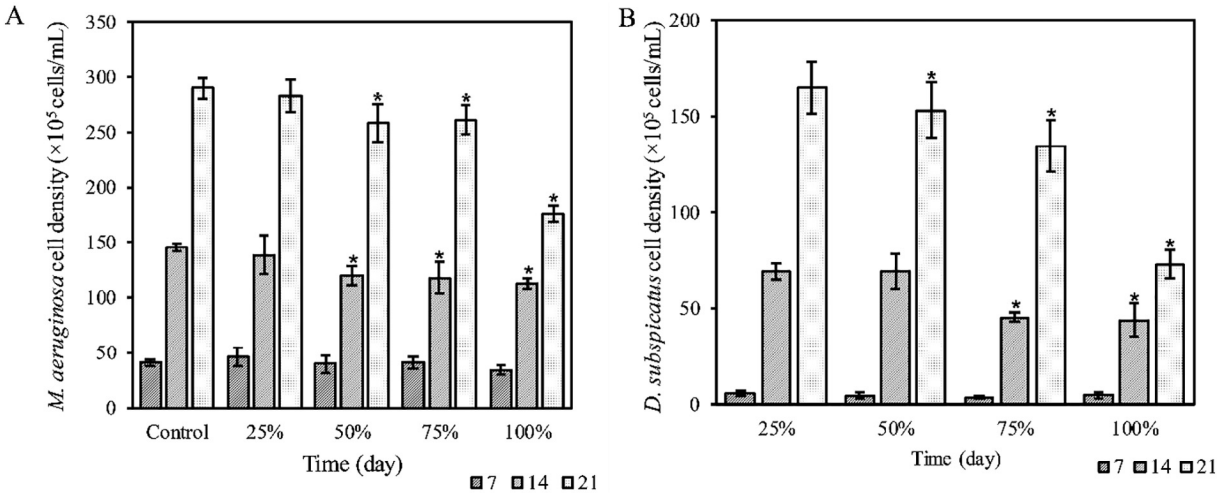

Fig. 4. The growth of A) *M. aeruginosa* in cell-free spent medium of *D. subspicatus* and B) *D. subspicatus* in cell-free spent medium of *M. aeruginosa*, at different percentages of the filtrates (25, 50, 70, and 100%). Data represent mean values ± standard deviation ($n = 5$). Significant differences observed at $p < 0.05$ (*).

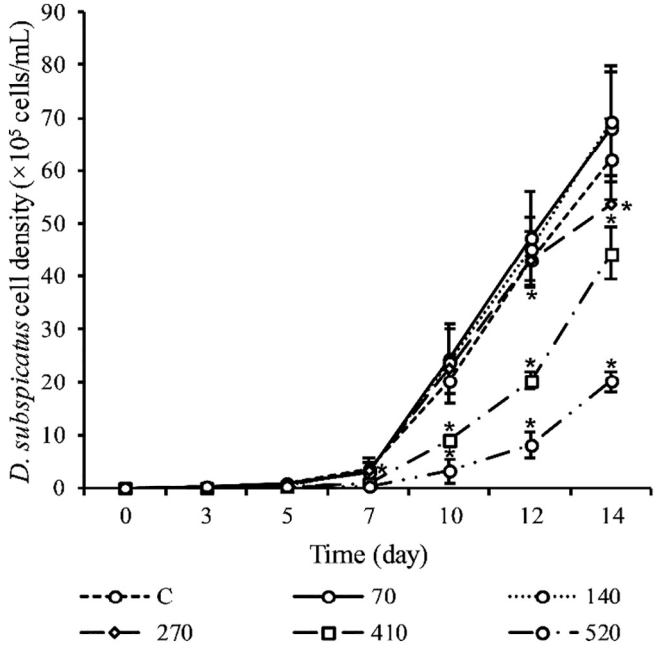

Fig. 5. The growth of *D. subspicatus*, which was exposed to the different concentrations of the extracted MC-LR from *M. aeruginosa* (70, 140, 270, 410, and 520 μg/L). Data represent mean values ± standard deviation (n = 5). Significant differences observed at $p < 0.05$ (*).

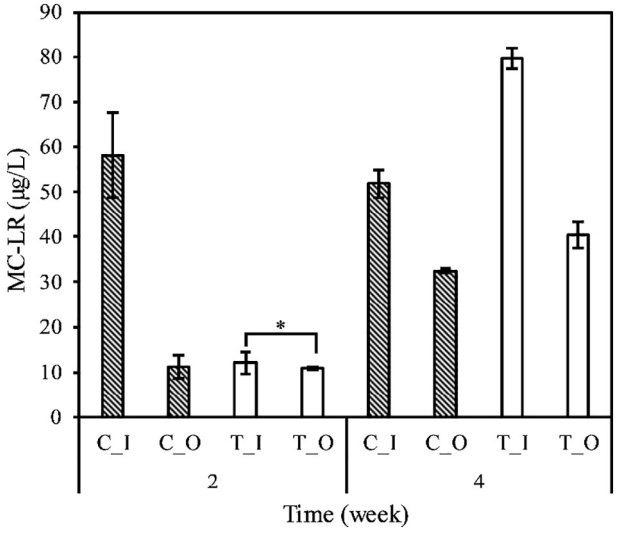

Fig. 6. The concentration of the extracellular MC-LR (μg/L) of *M. aeruginosa* at the inside and outside of the dialysis tubing in the controls and treatments (C: control, T: treatment, I: inner membrane, O: outer membrane). Data represent mean values ± standard deviation ($n = 3$). An asterisk (*) indicates no significant difference ($p > 0.05$) observed between the inner and outer membrane MC-LR of control and treatment.

fourth week, the smaller concentration of total extracellular MC-LR in monocultures (84.3 μg/L) has been better equilibrated (Fig. 7A). On the other hand, the worst diffusion rate (0.2) has been observed in the monocultures of the second week, which contained a lower concentration of total extracellular MC-LR, compared to the control and treatments of the fourth week (Fig. 7B).

5. Discussion

In the present study, a co-cultivation system was designed which composed of two microalgae populations separated physically using a dialysis membrane, thereby still allowing the two to affect each other via their diffusible extracellular products beyond their physical associations. Over time, the inner membrane growing species, *M. aeruginosa*, released an increasing concentration of MC-LR, which diffused through the membrane and was detected at both sides of the tubing. The results indicated that at the lowest concentration of extracellular MC-LR, it could be equally distributed between the inner and outer sides of the dialysis membrane. Moreover, the diffusion rate of the released MC-LR was negatively related to the total concentration of extracellular MC-LR.

Regarding the experimental design, MC-producing *Microcystis* was placed inside of the dialysis tubing to avoid the reduction of the light intensity, which might have resulted from the shadow effects of the buoyant *Microcystis* cells in the mixture. Furthermore, the competition for nutrients was prevented according to the suggestion by Dunker et al. (2013) and Bittencourt-Oliveira et al. (2015). The species were cultivated in BG 11 medium which is a nutrient rich growth medium at the equal initial cell density (1,1), and fresh nutrient was added regularly to avoid giving one species the advantages of the higher initial biomass or the different ability in nutrient uptake to dominate the mixed culture. However, nutrient concentrations were not measured during the co-cultivation experiments for undoubted rejection of the possibility of resource competition between the co-cultured species.

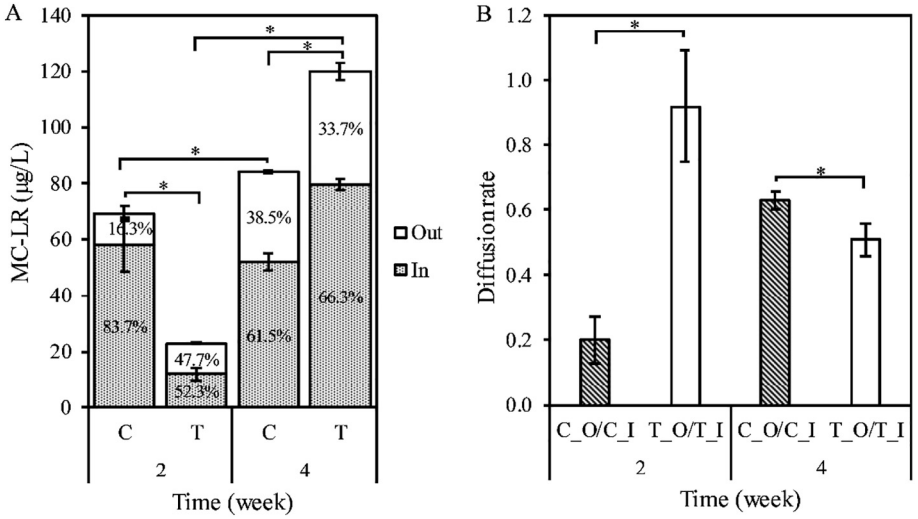

Fig. 7. The percentage (A) and the diffusion rate (B) of the extracellular MC-LR in mono and co-cultures at the 2nd and 4th week (C: control, T: treatment, I: inner membrane O: outer membrane). Data represent mean values ± standard deviation (n = 3). Significant differences observed at $p < 0.05$ (*).

The results indicated that in the co-cultivation system, both species affected one another depending on the growth stages. At the early logarithmic phase of growth, the presence of *Microcystis* did not influence the growth of green alga, while the green alga started to outcompete the co-cultured *Microcystis* which was further inhibited during the exponential phase of growth. The inhibitory effects of the green algae and some species of cyanobacteria on the growth, MC production and photosynthesis of *Microcystis* spp. was reported in other investigations through the reduction in chlorophyll *a* content (Jia et al., 2008), the production of bioactive compounds capable of inhibition of MC production and increasing of the activity of alkaline phosphatase (ALP) (Rzymski et al., 2014), disturbing of the synthesis of proteins and polysaccharides and chlorophyll *a* content (Qiu et al., 2019), induction of cell lysis (Harel et al., 2013; Bittencourt-Oliveira et al., 2015), and the greater nutrient uptake ability of the green algae (Huan et al., 2006). Moreover, the results indicated that the reduction rate of MC release in co-cultures was at the same level as the decreased rate of the biomass of *Microcystis*. Therefore, the reduction of the extracellular MC-LR was due to the decreased *M. aeruginosa* cell density, and the decreased total MC content have resulted from the significant suppression of the MC synthesis. Since the MC production rate is positively related to the cell division rate, it can be assumed that the growth inhibition and suppression of MC synthesis might be related to the limiting factors such as light intensity and interferences in the nutrient uptake capability of *Microcystis* (Orr and Jones, 1998; Lyck, 2004; Downing et al., 2005; Deblois and Juneau, 2010; Chaffin et al., 2018; Kramer et al., 2018). MC production is highly depended on the nitrogen supply (Harke and Gobler, 2013, 2015). The binding site of NtcA, a global nitrogen regulator in cyanobacteria, was found in the promoter region of the mcy gene cassette (Ginn et al., 2010).

On the other hand, the study by Huan et al. (2006) demonstrated that in a mixed culture, the growth of *M. aeruginosa* was inhibited due to the greater ability of the green alga *Chlorella ellipsoidea* in the utilisation of nitrogen and phosphorous. Zhang et al. (2013) found that the green algae *Quadrigula chodatii* FACHB-1080 inhibited the growth of *M. aeruginosa* PCC7820 through the production of allelochemicals such as dibutyl phthalate and beta-sitosterol, and the interferences in the nitrogen uptake and utilisation by *Microcystis*. In the current study, the culture filtrate of the green alga negatively affected the growth of *Microcystis*, which might reinforce the probable interferences of the extracellular metabolites of the green alga in out-competing of the co-existing *Microcystis*. Moreover, the inhibition of the growth of *Microcystis* was observed earlier in co-cultures (after the first week) than compared to exposure with the *Desmodesmus* filtrate, which might be due to the interferences of the green alga in nutrient uptake capability of *Microcystis* in co-cultures. However, it is important to point out that the MC production rate is regulated by light intensity (Deblois and Juneau, 2010; Renaud et al., 2011). *With the progression of the experiment,* light limitation may have occurred due to the *shading of the dense algal cultures, especially at the logarithmic phase of growth,* which should be considered in future studies.

Over time, towards the stationary phase of growth, in co-cultures, MC production and release were increased, coinciding with the inhibition of the growth of the green alga. However, the exposure of the green alga to the extracted MC-LR showed that the growth of green alga was inhibited only at MC concentrations that were greater than the outer membrane MC concentration (79.5 μg/L) of the co-culture experiments. Past studies have shown that the exposure of aquatic organisms to the *Microcystis* crude extracts caused greater activity in detoxification enzymes in the target species, compared to the purified MCs and the intact cells of *Microcystis* due to the presence of the other toxin modulating factors (Pietsch et al., 2001; Vasconcelos et al., 2007; Scoglio, 2018). However, the interspecies interference between intact cells in a consistent mode of microbial exposure might be more complicated. On the other hand, regarding the inhibitory effects of the *Microcystis* spent medium on the growth of green alga, it might be assumed that the other probable extracellular metabolites of *Microcystis* might be involved in the interspecies interplay. *Microcystis* is known to produce the other secondary metabolites such as micropeptin, microviridin, microgenin, as well as some unknown compounds that might be involved in the interferences of cyanobacteria with other phytoplankton species (Ikawa et al., 1996; Reshef and Carmeli, 2001; Ploutno et al., 2002). However, the probable synergistic effects between other metabolites and MC-LR to outcompete the co-existing species should be considered.

Previous studies have shown that in a mixed culture, *M. aeruginosa* severely inhibited the growth of the green algae *Chlorella pyrenoidosa* (Hong et al., 2010), and the growth and photosynthesis of the dinoflagellate *Peridinium gatunense* through abolishing carbonic anhydrase activity (Sukenik et al., 2002). Furthermore, the growth and photosynthesis of other aquatic organisms which were exposed to the purified MCs (25–50 μg/mL) or the crude extracts of *Microcystis*, were inhibited due to the reduction of CO_2 uptake, depletion of nitrogen fixation (Singh et al., 2001), and promotion of the oxidative stress (Pflugmacher, 2004; Paskerová et al., 2012). Therefore, at the exponential phase of growth, the growth of green alga was not altered,

perhaps due to the repair systems of the green alga such as anti-oxidative enzymes (Cirulis et al., 2013). The study by Mohamed (2008) showed that MCs had been absorbed and biotransformed in the green algae, *Chlorella* and *Scenedesmus*. Additionally, it is speculated that the green alga could produce intra- and extracellular polysaccharides as an adaptive response to protect the cells against the oxidative stress (Mohamed, 2008; El-Sheekh et al., 2012). However, by entering the stationary phase of growth, the increased release of metabolites from *Microcystis* due to the increased lysis of *Microcystis* cells might have resulted in the inhibition of the growth of the green alga through the interferences in the photosynthesis process, inhibition of serin protease activity and the induction of oxidative stress (Smith et al., 2008).

On the other hand, the results of the co-cultivation experiments indicated that the rate of enhanced toxin release in co-cultures was significantly higher than the elevated rate of cell density. While in monocultures, they have enhanced approximately at the same level. Previous studies showed that the MC production and release could be induced under stress conditions such as the limited nutrient availability caused by the presence of the competitor species (Kaplan et al., 2012; Pimentel and Giani, 2014; Yeung et al., 2016). Additionally, the extracellular metabolites of green algae could induce MC production in *M. aeruginosa* (Bittencourt-Oliveira et al., 2015), and cause disruption of *Microcystis* cell membrane at the stationary phase of growth (Harel et al., 2013).

In summary, applying a co-cultivation system allowed investigating the interspecies interference between intact cells in a consistent mode of microbial exposure to simulate the natural ecosystems where microorganisms are co-existing within various microbial communities. However, the natural ecosystems are much more complicated than the laboratory-controlled conditions. A combination of the conventional exposure of target species to cellular exudates, purified metabolites such as MCs and co-cultivation studies might provide more insights into the probable mechanisms of the interspecies interplays, through the resource competitions, the interferences of the bio-compounds, or a combination of both. Moreover, the characterisation of the probable bioactive compounds of the green alga might open the ways for the control of harmful cyanobacterial blooms or minimise the harmful effects of the release of a high concentration of MCs into the natural ecosystem that come into contact with the aquatic species following toxic cyanobacterial bloom degradation. With this in mind, it should be considered whether exudates of green alga are non-toxic to non-target organisms.

6. Conclusions

This study highlights the potential of the dialysis membrane to determine the interspecific interactions between the intact cells of the co-growing species. It can be used in future laboratory or field studies by the positioning of the dialysis membrane containing the individual species or the mixed samples in the natural ecosystems of the occurring cyanobacterial blooms.

It should be considered that the interspecies interactions through the bioactive compounds or due to the competition for the resources might influence the dynamics of the phytoplankton community. Both species might produce metabolites to influence the growth of each other negatively. The inhibition of the green alga at a concentration higher than the environmentally relevant concentrations of MCs (1–10 μg/L) might explain the co-existence of the cyanobacteria and green algae in the phytoplankton community. MC might improve the fitness of *Microcysts* cells under the stress conditions induced by the presence of the green alga. However, the inhibitory effects of *Microcystis* might be related to other probable extracellular metabolites of *Microcystis* in addition to MC. The presence of MC may reinforce the inhibitory effects of the *Microcystis* on the growth of the co-existing green alga which should be considered in future studies.

Data availability

All data reported in the present study are available via the Supplemental Data files or by request from the authors (stephan.pflugmacher@helsinki.fi).

Declaration of Competing Interest

None.

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